



PATENT
CL-213

APPLICATION FOR UNITED STATES PATENT

Title: HUMAN ANTI-TNF ANTIBODIES

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BACKGROUND OF THE INVENTION

Field: This disclosure relates generally to monoclonal antibodies and specifically with human antibodies that bind to human tumor necrosis factor (TNF α).

Prior Art: TNF α is a pluripotent and pleiotropic cytokine. It is produced principally by activated macrophages, however its synthesis and secretion have also been observed using granulocytes, tonsil B cells, B cell lines, natural killer cells, T cell lines, primary chronic malignant B cell isolates, and peripheral blood T cells.

TNF α can also be expressed on cell surfaces, apparently in two forms. One is a 26 kd molecular weight integral type 2 transmembrane protein on monocytes, T cells and some other cells. The other form is the secreted 17 kd product which is bound to its receptor.

Among the many activities of secreted TNF α are thymocyte growth factor, B cell growth and maturation factor, production in vivo of hemorrhagic necrosis, weight loss, cardiovascular collapse and multiple organ failure. Naturally, these latter activities are the source of the clinical interest in TNF α .

During septic shock, as well as inflammatory diseases, synthesis and secretion of TNF α , IL-1, IL-6 and IL-8 have been documented. Hence the immune systems of some individuals are exposed chronically to these cytokines. Indeed, low affinity antibodies to TNF α have been reported (A. Fomsgaard et al "Auto-antibodies to Tumor Necrosis Factor α in Healthy Humans and Patients with Inflammatory

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3 Diseases and Gram-Negative Bacterial Infections." Scand. J.
4 Immunol. 30:219-23, 1989; and, K. Bendtzen et al "Auto-
5 antibodies to IL-1 α and TNF α in Normal Individuals and
6 Infectious and Immunoinflammatory Disorders." Prog.
7 Leukocyte. Biol. 10B:447-52, 1990). These anti-TNF α
8 autoantibodies may, however, not be specific (H.-G. Leusch
9 et al "Failure to Demonstrate TNF α Specific Autoantibodies
10 in Human Sera by ELISA and Western Blot." J. Immunol. Meth.
11 139:145-147, 1991).

12 One peculiar feature of human serum, as well as sera
13 from other animals, is its content of natural and so-called
14 polyreactive antibodies. These are usually IgM antibodies
15 which bind to various autoantigens with low affinity (A.B.
16 Hartman et al "Organ Reactive Autoantibodies from Non-
17 Immunized Adult Balb/c Mice are Polyreactive and Express
18 Non-Biased Vh Gene Usage." Molec. Immunol. 26:359-70, 1989;
19 and, P. Casali et al "CD5+ B Lymphocytes, Polyreactive
20 Antibodies and the Human B cell Repertoire." Immunol.
21 Today. 10:364-8, 1989). Hence the autoantibody-like
22 reactivity to human TNF α might be expected to be low
23 affinity and probably cross-reactive with several other
24 antigens.

25 Some high affinity neutralizing antibodies to IL-1 α
26 have been reported in normal sera (N. Mae et al
27 "Identification of High-Affinity Anti-IL-1 α Autoantibodies
28 in Normal Human Serum as an Interfering Substance in a
Sensitive Enzyme-Linked Immunosorbent Assay for IL-1 α ."
Lymphokine Cytokine and Research 10:(1)61-68, 1991) or
patient (H. Suzuki et al "Demonstration of Neutralizing
Autoantibodies Against IL-1 α in Sera from Patients with
Rheumatoid Arthritis." J. Immunol. 145:2140-6, 1990).

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3 Despite these considerations, we are unaware of the
4 disclosure of any monoclonal human antibodies specifically
5 binding to TNF α even though it is thought such antibodies
6 may have significant clinical value. Thus, there has
7 remained a need for monospecific monoclonal antibodies to
8 TNF α .

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10 SUMMARY OF INVENTION:

11 We have made monoclonal human antibodies which bind to
12 both human and mouse TNF α . The antibodies bind to
13 recombinant human TNF α (rhTNF α) with a titer comparable to
14 three high affinity neutralizing mouse mAbs, when tested by
15 ELISA. The antibodies most fully characterized are of the
16 IgM isotype although we also prepared antibodies of the IgG
17 isotype. By competition binding experiments, the antibody
18 appears to bind to epitopes on rhTNF α distinct from those
19 bound by the neutralizing mouse mAbs so far described.
20 Specificity analyses indicate that the human IgM
21 autoantibody binds to both human and mouse recombinant TNF α ,
22 but not to other antigens commonly recognized by
23 polyreactive natural IgM autoantibodies. The high level of
24 amino acid identity between the human and mouse TNF α
25 molecules suggest that the antibody is monospecific for a
26 given epitope shared by these two forms of TNF α .

27 The B5 antibody also binds to cell surface TNF α (cs
28 TNF α) on human T cells, B cells, monocytes, a variety of
lymphoid and monocyte lineage cell lines of human origin, as
well as astrocytomas, a breast carcinoma, and a melanoma.
The antibody also binds to chimpanzee lymphocyte and mouse T
lymphoma cell line csTNF α . Binding of the antibody to
csTNF α is specific since it can be inhibited by TNF α but not

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3 by $\text{TNF}\beta$, a neutralizing mouse anti- $\text{TNF}\alpha$ mAb, nor by a
4 recombinant form of the extracellular domain of the p55 TNF
5 receptor (TNFR). The B5 autoantibody can inhibit LPS
6 induced $\text{TNF}\alpha$ secretion by cells of the human monocyte-like
cell line THP-1.

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8 Several monoclonal mouse anti-human $\text{TNF}\alpha$ antibodies
9 have been described in the literature. None, however, also
bind to mouse $\text{TNF}\alpha$.

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11 The specificity, the autoantibody nature, the binding
12 to cell surface $\text{TNF}\alpha$ and the ability to inhibit $\text{TNF}\alpha$
secretion make B5 a novel mAb.

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14 Characterization of the antibodies and how to make them
15 are described below.

16 BRIEF DESCRIPTION OF THE FIGURES

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18 Figures 1A and 1B show, in graph format, a comparison
19 of solid phase ELISA format binding to rh $\text{TNF}\alpha$ of the B5
20 (human) and A10G10 (murine) monoclonal antibodies. ELISA
21 plates were coated with various concentrations of TNF and
22 titrated doses of mAb were then allowed to bind. Shown are
the binding curves for each antibody for the various TNF
coating concentrations.

23
24 Figures 2A and 2B show, in graph format, the lack of
25 competition for binding to $\text{TNF}\alpha$ between mouse mAbs and B5
26 mAb. Figure 2A shows the binding of three mouse anti-TNF
27 mAbs and the control C7F7 anti-rFVIII mAb binding to solid
28 phase rh $\text{TNF}\alpha$. Figure 2B shows the lack of inhibition of B5
binding to plate bound TNF when the mouse monoclonals are

first allowed to bind to TNF plates and B5 antibody is added subsequently.

Figure 3 shows, in bar graph format, the binding of human IgM anti-TNF mAbs to rhTNF α captured and presented as a complex by the combination of plate bound mouse mAbs A10G10, B6 and A6. ELISA plates were precoated with the three mouse mAbs and then incubated with rhTNF α . Plates were washed and 20 ug/ml of the indicated human IgM mAbs were then allowed to bind. Solid bars show the binding of the human IgM mAbs to the three mouse mAbs which had been incubated with TNF, the hatched bars show binding of the IgM mAbs when the attached mouse mAbs had not been exposed to TNF.

Figures 4A-4F show, in multiple graph format, results of an analysis of the binding specificity of several monoclonal antibodies. Plates were precoated with either recombinant human TNF α (■), recombinant human lymphotoxin (◆), human insulin (□), porcine thyroglobulin (▲), BSA (○), ssDNA (■), dsDNA (□) or human IgG Fc fragments (Δ). Mouse mAb A10G10 is shown in panel A. Human IgM mAbs B5, 7T1, H5, 1A6B5F and F2.2.34 are shown in panels B, C, D, E and F, respectively. Antibody binding was assessed by ELISA.

Figure 5 shows, in graph format, binding of B5 to recombinant mouse TNF α . Plastic plates were precoated with a neutralizing monoclonal hamster anti-mouse TNF α antibody at 8 ug/ml (squares), 4 ug/ml (triangles) and 2 ug/ml (circles). Recombinant mouse TNF α was then added at 2 ug/ml (filled symbols) or was not added (open symbols). Human mAb B5 was then allowed to bind at the concentrations indicated.

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3 Binding was then assessed by ELISA using anti-human IgM
4 antibody.

5 Figure 6 shows, in graph format, a comparison of B5 mAb
6 (triangles) and mAb A10G10 (circles) binding to soluble
7 rhTNF α . Antibodies were bound to plastic plates precoated
8 with anti-human or anti-mouse antibody. Biotinylated TNF
9 was then incubated with the antibodies. Binding of soluble
10 TNF α was detected by enzyme-avidin conjugates.

11 Figure 7 shows, in graph format, that captured B5 mAb
12 binds soluble TNF α and weakly presents it to A10G10 mAb. B5
13 mAb anti-TNF α or 6F11 (human anti-LPS IgM) as a control,
14 were allowed to bind to plates precoated with anti-human
15 IgM. Soluble TNF α was then allowed to bind to the complexed
16 human mAbs. Mouse mAb A10G10 was added and its binding to
17 TNF complexed to B5 mAb was detected by enzyme linked anti-
18 mouse IgG antibody.

19 Figure 8 shows, in photograph format of Western blots,
20 the binding of several human IgM antibodies to mouse TNF α
21 and binding of the human B5 mAb to human TNF α . Recombinant
22 mouse TNF α (lanes A-G) and rhuTNF α (lanes H and I) were
23 electrophoresed under reducing conditions and transferred to
24 nitrocellulose. Mouse TNF α was blotted with the following
25 monoclonal antibodies: 7T1 (lane A), B5 (lane B), 1A6B5F
26 (lane C), 6F11 (lane D), H5 (lane E), A8 (lane F), and no
27 primary antibody (lane G). Human TNF α was electrophoresed
28 in lanes H and I. Lane H was then blotted with B5 mAb and
lane I with 6F11 mAb. Lanes A-F, H and I were then exposed
to biotinylated anti-human IgM. Lane F was exposed to
biotinylated anti-human IgG, since A8 is an IgG antibody.
All lanes were then exposed to the developing reagent avidin

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3 coupled horse radish peroxidase. Molecular weight
4 standards, ranging in molecular weight from 211 kd to 15.4
5 kd, were run in parallel and their positions are indicated.

6 Figure 9 shows, in graph format, the neutralization of
7 rhTNF α by A10G10 mouse mAb and lack of neutralization by
8 human mAbs. WEHI 164 cells were incubated with a cytotoxic
9 dose of rhTNF α in the presence of titrated concentrations of
mAb. Viability was subsequently assessed.

10 Figures 10A-10H show, in histogram format, the
11 fluorescence staining profiles of two cell lines stained
12 with human IgM anti-TNF α mAbs. 8B9 cells (Figure 10A, 10C,
13 10E, 10G) and THP-1 cells (Figure 10B, 10D, 10F, 10H) were
14 stained with no antibodies (Figures 10A, 10B), with FL-
15 F(ab)'₂ anti-human IgM (Figures 10C, 10D), B5 IgM anti-
16 TNF α +FL-anti-IgM (Figures 10E, 10F) and 6F11 anti-LPS+FL-
17 anti-IgM (Figures 10G, 10H). Fluorescence intensity channel
18 numbers, in arbitrary units are plotted against the cells
19 per channel on the ordinate. For each sample 5000 cells
20 were accumulated. The percentages of cells falling within
the indicated markers, scored as fluorescence positive, are
given.

21 Figures 11A-11B show, in graph format, the detection of
22 cell surface expression of TNF α on THP-1 and U937 cells with
23 the B5 anti-TNF α mAb, and increase in expression with LPS
24 and PMA. THP-1 (Figure 11A) and U937 (Figure 11B) cells
25 were incubated 3 hours with medium (open circles), LPS
(filled circles) or LPS+PMA (filled triangles).

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27 Figures 12A-12D show, in graph format, the shift in
28 staining intensity when B5 anti-TNF α IgM mAb binds to cells

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3 being stained with F1-anti-IgM antibody. CD19 positive
4 splenocytes are shown. These were stained with
5 phycoerythrin conjugated anti-CD19 and only positive cells
6 were further analyzed for fluorescein conjugated antibody
7 staining. Figure 12A shows C19+ splenocytes not stained
8 with FL-anti-IgM. Figure 12B shows staining of these cells
9 with B5+FL-anti-IgM, figure 12C shows staining with FL-anti-
10 hIgM alone, and figure 12D shows staining with control 6F11
11 anti-LPS IgM+FL-anti-IgM. The percentages of cells within
12 the indicated markers are given, indicating the percentage
13 of cells staining positively with the fluorescein conjugated
14 antibody. The median channel numbers for the positive
15 populations are also given. These numbers reflect the
16 staining intensity, measured in arbitrary units, for the
17 florescence positive populations.

18 DETAILED DESCRIPTION OF INVENTION

19 Materials and Methods

20 Reagents: Bayer A.G., Wuppertal, Germany provided rhTNF α .
21 The rmTNF α and rhLT were purchased from Genzyme. Human IgG
22 Fc fragments were purchased from Chemicon. Insulin was
23 purchased from Novo Nordisk Labs and all the other antigens
24 used in ELISAs were purchased from Sigma. The Staph. aureus
25 Cowan strain was purchased from Calbiochem (San Diego, CA).
26 The anti-human IgD-Dextran conjugate was obtained from a
27 private source. Phorbol myristic acid, mouse IgG₁,
28 staphylococcal enterotoxin B (SEB) and phytohemagglutinin
(PHA) were purchased from Sigma. E. coli LPS was obtained
from a private source. The different fetal bovine sera
(FBS) were purchased from Hyclone.

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3 The cell lines mentioned in Table 2 were all purchased
4 from the American Type Culture Collection (ATCC), except for
5 the 8B9 EBV transformed human B cell line which was obtained
6 from Genetic Systems Corporation.

7 TNF was biotinylated using standard techniques;
8 briefly, N-hydroxysuccinimidyl ester of biotin was added to
9 TNF dissolved in 50 mM NaHCO₃, pH 8.5 for 15 min, quenched
10 with NH₄Cl then dialyzed to remove unreacted biotin.

11 The mouse A10G10 anti-TNF α IgG₁ mAb was generated in
12 collaboration with Chiron Corporation and has an ATCC
13 designation number HB 9736, identified as hybridoma cell
14 line 2-2-3E3.

15 The A6 and B6 mouse IgG₁ mAb were generated from mice
16 hyperimmunized in our laboratory. All three mouse mAbs
17 neutralize TNF cytotoxicity and have been described in
18 Galloway et al "Monoclonal anti-Tumor Necrosis Factor (TNF)
19 Antibodies Protect Mouse and Human Cells from TNF
20 cytotoxicity." J. Immunol. Meth. 140:37-43, (1991) which is
21 incorporated herein by reference. These mAbs were purified
22 by affinity chromatography.

23 The polyreactive IgM mAbs 1A6B5F and F2.2.34 were
24 produced and characterized by Kasaian et al "Identification
25 and Analysis of a Novel Human Surface CD5- B Lymphocyte
26 Subset Producing Natural Antibodies." J. Immunol. 148:2690-
27 702 (1992). The 7T1 human IgM mAb was produced and provided
28 in ascites by a private source.

The 6F11-E4 (6F11) EBV transformed B cell
lymphoblastoid line having ATCC designation number CRL 1869,

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3 produces a human anti-Fisher type 2 Pseudomonas LPS specific
4 IgM antibody and was purchased from Genetic Systems
5 Corporation. The monoclonal antibody from this cell line
6 was produced in our laboratory. It serves as an isotype
7 matched control mAb for the human anti-rhTNF α mAbs. The
8 C7F7 mAb is a mouse IgG₁ anti-hFVIII developed in
9 collaboration with Genentech Inc. and is used as a isotype
10 matched control mAb for the mouse anti-rhTNF α mAbs.

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12 Goat anti-mouse IgG and biotinylated goat anti-human
13 IgG were purchased from Jackson Labs. Biotinylated goat
14 anti-mouse IgG and biotinylated mouse anti-human IgM were
15 purchased from Zymed. Avidin coupled HRP and avidin coupled
16 alkaline phosphatase were purchased from Zymed.

17
18 Phycoerythrin conjugated anti-CD3 and anti-CD19
19 antibodies were purchased from Dakopatts. Phycoerythrin
20 conjugated anti-LeuM3 was purchased from Becton Dickinson.
21 Fluorescein (FL) conjugated F(ab)'₂ anti-human IgM, FL-
22 F(ab)'₂ anti-human IgG and FL-F(ab)'₂ anti-mouse IgG
23 antibodies were purchased from Cappel.

24
25 ELISAs: Antigens or capture antibodies (anti-immunoglobulin
26 antibodies) were coated to plastic plates in
27 carbonate/bicarbonate buffer, or PBS containing 20 ug/ml
28 BSA, overnight at 4°C or 3 hrs at 37°C. Secondary
incubations were carried out overnight at 4°C or at room
temperature for a period of 2 hrs or less. Secondary
antibodies were biotinylated and their binding was revealed
using avidin coupled HRP and avidin coupled alkaline
phosphatase.

SPECIFIC EMBODIMENTS

Hybridoma Production: The human IgM mAbs were produced by fusion with the mouse P3X63Ag8.653 non-secreting myeloma. Peripheral blood mononuclear cells from a CMV positive donor were separated by centrifugation on Ficoll, treated with L-leucyl leucine methyl ester, incubated in vitro with antigen and subsequently transformed with EBV. Transformants were distributed at limiting concentrations and cells producing antibody binding to TNF were fused and subsequently subcloned. The B5 hybridoma was subcloned a minimum of 5 times and was deposited with ATCC on MARCH 24, 1993 as deposit CHL11306. The H5 and 7T1 mAbs were produced by fusion of human tonsillar cells immunized in vitro. Monoclonal human IgM antibodies were affinity purified by standard techniques for use in subsequent experiments.

Cytotoxicity Assay: To assess the TNF neutralizing ability of various mAbs, the assay described by Galloway et al (cited above) was used with the following minor modifications. Briefly, 20 pg/ml TNF were incubated overnight with 60,000 WEHI 164 cells and the test mAb. Viable cells were then detected by crystal violet staining and reading optical density at 570 nm.

Western Blotting: Recombinant huTNF α (100 ug/ml plus 100 ug/ml BSA) and recombinant mTNF α (5 ug/ml with 100 ug/ml BSA) were electrophoresed in the presence of β mercaptoethanol and SDS on 12% polyacrylamide gels. Proteins were then electro-transferred to nitrocellulose which was then blocked with BSA. Test mAbs were allowed to bind and were subsequently detected with biotinylated anti-

immunoglobulin reagents. Streptavidin-HRP was then added followed by substrate.

Fluorescence Analyses: One million cells were stained with optimal concentrations of primary antibody, usually 2.5-40 ug/ml at 4°C for 1/2 hour in PBS containing 1% FBS and 0.02% sodium azide. Optimal concentrations of fluorescent secondary antibodies were added, after two cell washes, for a similar time in similar buffer. After washing, cells were fixed with 2% paraformaldehyde solution. Cell fluorescence was then analyzed on a FACSCAN (name of instrument).

Inhibition of LPS stimulation of TNF α Secretion: One million THP-1 cells/ml were incubated 4 hrs with 1 ug/ml E. coli LPS in the presence or absence of 40 ug/ml human IgM antibodies. Supernatants were harvested, centrifuged, filtered and assayed for TNF α cytotoxicity in the WEHI 164 assay mentioned above. Supernatants were titrated and viability was plotted against supernatant dilution. These curves were compared to a standard curve using rhTNF α to determine the actual concentrations of TNF α produced by the cells.

Results

The monoclonal human IgM antibody B5 binds to solid phase recombinant human TNF (rhTNF α). Several hybridomas secreting monoclonal anti-rhTNF α antibodies have been established in our laboratory. An endpoint titer analysis was performed comparing a panel of 6 human IgM mAbs and 3 human IgG mAbs to three high affinity neutralizing mouse mAbs, A10G10, A6 and B6. ELISA plates were coated with 2 ug/ml rhTNF α . The indicated mAbs were added in titrated

concentrations and binding was assessed spectrophotometrically. The minimum mAb concentrations yielding detectable rhTNF α binding are shown. B5 and F12 (F80-1B9-F12) were two of the best human IgM mAbs by this criterion, showing endpoint titers in the subnanogram/ml range. Table 1 presents the data below.

Table 1
Comparison of Solid Phase ELISA Format
rhTNF α Binding by Several Monoclonal Human Antibodies

mAb	Endpoint Titer (ng/ml)	Ig Class
A1-G10	0.6	mouse IgG
A6	0.15	mouse IgG
B6	0.08	mouse IgG
F78-1A10-A1	0.3	human IgM
F78-1A10-B5	0.6	human IgM
F80-1B9-F12	0.15	human IgM
F81-4E3-D6	9.8	human IgM
F83-1D6-B6	625.0	human IgM
D83-1D6-F6	1250.0	human IgM
F83-1A7-G7	0.76	human IgG
F83-1G12-C1	1.5	human IgG
F83-4D3-D8	0.38	human IgG
F83-8D5-F10	0.76	human IgG
F84-6G9-D6	1563.0	human IgG

It should be noted that the ranges and endpoint titers were similar for the IgM anti-TNF α mAbs and the IgG anti-TNF α mAbs.

Figure 1 presents a more extensive comparison of the human B5 and mouse A10G10 mAbs. Binding of both mAbs was concentration dependent regardless of TNF coating

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2
3 concentration. The B5 mAb bound slightly better than A10G10
4 with high TNF coating concentrations. As the TNF coating
5 concentration was reduced, however, the binding of B5
6 decreased more rapidly than that of A10G10. This is
7 consistent with B5 having a lower affinity than A10G10 for
8 rhTNF α . These data show that the B5 mAb binds to solid-
9 phase rhTNF α .

10 B5 mAb binds to a different epitope on rhTNF α than
11 those bound by three mouse anti-TNF mAbs. Competitive
12 binding experiments have shown that A10G10 and B6 recognize
13 similar epitopes on rhTNF α whereas A6 recognizes a different
14 epitope (data not shown). To examine the epitope binding
15 specificity of B5, competitive binding experiments were
16 performed using the mouse mAbs and B5.

17 The mouse mAbs were added at different concentrations
18 to ELISA plates previously coated with TNF α . An optimum
19 concentration of B5 mAb was then added and binding was
20 subsequently detected with biotinylated anti-human IgM. If
21 the mouse mAbs recognize the same epitope as B5 mAb, they
22 should inhibit B5 mAb binding in a concentration dependent
23 manner.

24 As shown in Figure 2A, binding of the mouse mAbs to
25 plate bound rhTNF α is concentration dependent. Figure 2B
26 shows that none of the mouse mAbs interfered with rhTNF α
27 binding by a fixed amount of B5 mAb, even at concentrations
28 of the mouse mAbs significantly in excess of those required
for maximal binding to the plate. These data suggest that
B5 recognizes an epitope on rhTNF α different from those
recognized by A10G10, A6 and B6.

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3 To support this finding, rhTNF α was added to ELISA
4 plates previously coated with the combination of A10G10, B6
5 plus A6 mAbs. B5 mAb was then added to test whether it
6 could bind to rhTNF α complexed to, or captured by, the mouse
7 mAbs.

8 Figure 3 shows that B5 and all the other human IgM
9 mAbs, except 7T1, bound to rhTNF α complexed to mouse mAbs.
10 Binding of the human mAbs was not seen in the absence of
11 rhTNF α , demonstrating specificity for some epitope of
12 rhTNF α . The failure of 7T1 mAb to bind to complexed TNF may
13 be simply due to a low affinity. These results support the
14 conclusion that the human IgM mAbs B5, F12, A1, B6 and D6
15 and the three mouse mAbs recognize different epitopes on
16 rhTNF α .

17 B5 mAb is not polyreactive. Since B5 mAb is a human
18 IgM which binds to human TNF α , and therefore has properties
19 which define it as an autoantibody, it was important to
20 determine the quality of this mAb and assess its
21 polyreactivity. We chose a panel of human and non-human
22 antigens typically used to define polyreactivity. Binding
23 of these antigens by B5 mAb, A10G10, two control
24 polyreactive human IgM mAbs 1A6B5F and F2.2.34 and two other
25 human IgM anti-TNF mAbs was compared. The results have been
26 normalized for each antibody to allow direct comparison.

27 Figure 4 presents the data from one of four similar
28 experiments. The mouse mAb A10G10 binds specifically to
rhTNF α and none of the other antigens. In contrast, the
polyreactive mAb 1A6B5F binds to virtually all of the
antigens tested. The same was true for the other
polyreactive mAb F2.2.34, although binding to BSA and TNF

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3 was much stronger than that seen with the other antigens.
4 The B5 mAb showed specificity for rhTNF α . No binding by B5
5 mAb to recombinant human lymphotoxin (rhTNF β) nor to any of
6 the other antigens tested was observed. These data provide
evidence that the B5 mAb is not polyreactive.

7
8 In contrast, the 7T1 and H5 human IgM mAbs bind to
9 human Fc fragments indicating a rheumatoid factor nature.
10 These two antibodies also bind to insulin and 7T1 binds BSA
11 as well. The control polyreactive mAbs appear to define two
12 classes of polyreactivity; one being very broad in
13 specificity and the other being more restricted in the
14 antigens recognized. The 7T1 and H5 mAbs belong to the more
restricted class of polyreactive mAbs. The F12 anti-TNF mAb
binds to human TNF α but only marginally to other antigens.

15 B5 mAb binds to recombinant mouse TNF α . During the
16 course of analyzing the specificity of the B5 mAb, we
17 noticed that it also bound to mouse TNF α . To demonstrate
18 this, we first captured mouse TNF α with a neutralizing
19 hamster monoclonal antibody and then allowed B5 to bind to
20 this complex. Figure 5 shows the results of this kind of
21 experiment. The binding of B5 was dependent on both the
22 concentration of B5 present, and on the concentration of
23 hamster antibody used to coat the plates. No binding was
24 observed when mouse TNF α was not added, indicating the
25 specificity of B5 binding in this system. Other experiments
26 not shown revealed binding to mouse TNF α by the F12 mAb.

27 B5 mAb binds to soluble rhTNF α with detectable but low
28 affinity. Next, we assessed the mAb's ability to bind to
soluble rhTNF α . ELISA plates were coated with anti-human IgM

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3 and B5 was then added. The ability of the bound B5 mAb to
4 capture biotinylated rhTNF α was then determined.

5 Figure 6 compares the abilities of A10G10 and B5 to
6 bind soluble TNF α under these conditions. Although both
7 mAbs bind soluble rhTNF α , about 300-fold higher
8 concentration of B5 mAb is required for binding equivalent
9 to that of A10G10. Furthermore, binding of soluble TNF α to
10 immobilized B5 did not saturate with the concentrations of
11 B5 tested. These results are consistent with a low affinity
12 binding of rhTNF α by B5 mAb. Indeed, attempts to measure
13 the binding constant of B5 mAb revealed an affinity too low
14 to calculate by conventional methods (data not shown).

15 Soluble rhTNF α binding by B5 was also demonstrated by
16 coating plates with anti-IgM, capturing B5 and then adding
17 unmodified soluble rhTNF α . A10G10 was added next and its
18 binding to this B5-complexed form of rhTNF α was detected
19 with biotinylated anti-mouse IgG. Figure 7 compares the
20 abilities of B5 and a control human IgM, 6F11, to capture
21 and present soluble rhTNF α to A10G10. Although some non-
22 specific binding was seen with the control mAb, B5 mAb bound
23 approximately four- to eight-fold more rhTNF α in this
24 experiment. These data are consistent with a low binding
25 constant of B5 and add further support for the concept that
26 B5 mAb and A10G10 mAb recognize different epitopes on
27 rhTNF α .

28 B5 mAb recognizes rhTNF α in Western blots. Figure 8
shows the results of an experiment using western blotting to
demonstrate B5 binding to denatured TNF α . The images have
been enhanced for clarity. In lanes A-G, binding to mouse
TNF α was examined and in lanes H and I binding to human TNF α

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3 was examined. The 6F11 antibody did not bind to either TNF α
4 species and so provides a specificity control. All the
5 human IgM mAbs, 7T1, H5, 1A6B5F and B5 bind to mouse TNF α .
6 Furthermore, the B5 antibody also binds to human TNF α , under
7 these conditions. These results suggest that B5 may
8 recognize a linear epitope of rhTNF α .

9 B5 mAb does not neutralize the cytotoxicity of rhTNF α .
10 The TNF sensitive cell line WEHI 164 was used to assess the
11 ability of B5 mAb to neutralize TNF α cytotoxicity. Figure 9
12 shows that A10G10 clearly neutralizes rhTNF α in a dose
13 dependent manner as previously demonstrated by Galloway et
14 al (cited above). At no concentration of B5, however, was
15 any neutralization of rhTNF α observed. The same is true for
16 the three other human IgM anti-TNF α mAbs B6, F12 and 7T1
17 which were tested. These data add further support to the
18 idea that B5 and A10G10 bind different epitopes of TNF and
19 are consistent with the ability of B5 mAb to bind soluble
20 rhTNF α weakly.

21 The B5 mAb anti-rTNF α binds to the surface of several
22 different cell lines. Since the B5 mAb binds specifically
23 to rTNF α , several cell lines were chosen to test whether or
24 not the mAb would bind to their surfaces. Figure 10 shows
25 the results of a typical experiment using two cell lines.
26 The EBV transformed human B lymphoblastoid cell line 8B9 and
27 the human monocyte cell line THP-1 were stained with either
28 B5 anti-TNF α or the 6F11 anti-Pseudomonas LPS mAbs and then
fluorescent anti-human IgM F(ab)'₂ fragments.

The 8B9 cells were stained well with the B5 mAb whereas
no significant binding to the cell surface was seen with the
control 6F11 mAb. B5 staining was also observed with THP-1

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3 cells. However, fewer cells in this population were stained
4 and the observed staining was somewhat dimmer than that seen
5 for the 8B9 cells. Nevertheless, nearly 1/3 of the cells in
6 the THP-1 population expressed cell surface TNF α (csTNF α),
7 as detected with the B5 mAb. It is unclear whether this
8 level of staining reflects some regulation of csTNF α
9 expression or whether it is due to clonal variation within
10 the cell line.

11 The concentration dependence of B5 binding to cell
12 surfaces was examined more closely with the THP-1 monocyte
13 and U937 histiocyte cell lines. These cells were stained
14 with titrated amounts of B5 antibody after incubation with
15 either no stimulus, LPS or LPS+PMA for 3 hrs. The results
16 are shown in Figure 11. In all cases, B5 binding to cells
17 was dose dependent. Interestingly, more binding was
18 observed for both cell lines when they were preincubated
19 with LPS or LPS+PMA. This was especially apparent for the
20 U937 cell line. This increase is consistent with the known
21 ability of these agents to induce TNF secretion by monocyte
22 cell lines. Upon stimulation, B5 binding to the cells was
23 apparent, even at several hundred nanograms/ml of antibody.

24 Table 2 shows the results of two experiments in which
25 the binding of B5 anti-TNF α mAb was surveyed. Cells were
26 stained with the indicated primary antibodies and
27 fluorescein labeled anti-human IgM (μ -specific) secondary
28 antibody. The percentages of cells staining positively are
shown as determined on a FACSCAN instrument.

Table 2

Binding of the TNF α Specific B5 Human IgM mAb
to Various Cell Lines

		<u>% Cells Staining Positively</u> <u>primary antibody</u>			
Expt Line	Phenotype	none	B5	6F11	
1	8B9-EBV	human B lymphoblast	1.1	86.9	4.7
	1A2-EBV	human B lymphoblast	2.3	64.7	2.7
	hpbl-EBV	human B lymphoblast	2.0	96.2	2.3
	cpbl-EBV	chimpanzee B lymphoblast	6.6	76.1	6.2
	tonsil-EBV	human B lymphoblast	4.6	91.2	4.9
	Jurkat	human T lymphoma	0.7	17.9	1.2
	LBRM33	mouse T lymphoma	3.1	72.7	3.8
	DU4475	human breast carcinoma	10.2	52.4	9.8
	SW1088	human astrocytoma	11.2	15.3	10.9
	U118MG	human glioblastoma	6.2	7.3	6.2
	U373	human glioblastoma/ astrocytoma	4.9	69.6	3.5
2	U937	human histiocytic lymphoma	0.9	63.1	1.5
	THP-1	human monocyte	1.7	25.2	2.0
	1A2-EBV	human B lymphoblast	2.2	98.4	2.9
	8B9-EBV	human B lymphoblast	4.7	98.8	5.4
	A375	human melanoma	1.7	8.5	2.5

A variety of cell lines were tested including those of human B and T lymphocyte, breast carcinoma, astrocytoma, glioblastoma, monocyte, histiocyte, melanoma, and monoblast origin. A mouse T cell lymphoma was tested as well. Of the 15 lines tested, only the breast carcinoma U118MG showed no binding by B5. The others exhibited a range in the percentages of cells within each population which expressed cTNF α from a low of around 8% for the A375 melanoma to over 90% for EBV transformed B cells. The class matched 6F11 anti-LPS mAb failed to stain any of these cell lines. This

1
2
3 and the negative cell line indicate that the B5 staining
4 seen was specific and not the result of a general affinity
5 for all cells.

6 Lack of Neutralizing Mouse anti-TNF α mAb Binding to
7 cs TNF α

8 ELISA experiments have shown the TNF specificity of the
9 B5 mAb and demonstrated its binding to an epitope on TNF α
10 different from that bound by the neutralizing mouse mAb
11 A10G10. We next examined whether or not the epitope
12 recognized by A10G10 mAb was expressed on the surface of
cells to which B5 binds.

13 Table 3 presents data from five experiments addressing
14 this issue using the U937 and THP-1 cell lines. Cells were
15 stained with the indicated primary antibodies and
16 fluorescein labeled anti-mouse IgG (γ -specific) or anti-
17 human IgM (μ -specific) secondary antibodies. The asterisk
18 (*) indicates that F(ab)'₂ fragments of A10G10 mAb were
19 used. The percentages of cells staining positively are
20 shown as determined on a FACSCAN instrument. Not determined
21 is signified by nd.
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Table 3

Binding of Human B5 and Lack of Binding of Mouse A10G10
anti-TNF α mAbs to Cell Surface TNF on
Unstimulated Monocyte and Histiocyte Cell Lines

% Cells Staining Positively							
<u>Primary Antibody</u>							
Expt	Cell Line	none	A10G10	mIgG ₁	none	B5	6F11
1	U937	0.3	0.4	nd	2.9	15.1	2.7
2	THP-1	0.9	2.6	nd	2.3	24.8	2.8
3	U937	0.8	2.4	nd	2.7	99.1	2.8
4	THP-1	3.1	2.7	nd	2.7	34.7	3.4
5	U937	1.6	1.9	nd	1.6	35.7	1.8
6	THP-1	2.4	3.1	1.6	1.7	17.7	3.4
7	U937	2.8	2.9	2.8	2.2	20.2	2.7
8	THP-1	4.7	6.0*	6.7	4.6	56.3	nd
9	U937	1.5	9.9*	2.3	1.2	61.4	nd

In all five experiments the B5 mAb bound to each cell line. On the other hand, A10G10 mAb did not bind, to a significant degree, in four of the experiments. In one of the five experiments, a small amount of binding by A10G10 to the U937 cells was observed. Taken together, these data suggest that TNF α is on the surface of these cell lines, but the epitope recognized by A10G10 is only rarely available for binding by mAbs in the absence of exogenous stimulation.

LPS Induction of cell surface TNF α Expression.

LPS is a commonly used agent to induce TNF α secretion by human monocytes. We incubated THP-1 and U937 cells with LPS to examine whether or not csTNF α expression can be

increased. Table 4 shows the results of three experiments. Stimulation was performed by 3 or 4 hour incubation with 100 ng/ml LPS. Cells were stained with the indicated primary antibodies and fluorescein labeled anti-mouse IgG (γ -specific) or anti-human IgM (μ -specific) secondary antibodies. The asterisk (*) indicates that F(ab)'₂ fragments of A10G10 mAb were used. The percentages of cells staining positively are given as determined on a FACSCAN. Not determined is signified by nd.

Table 4
Analysis of Cell Surface Expression of TNF α After
Induction with Lipopolysaccharide

		<u>% Cells Staining Positively</u> <u>Primary Antibody</u>						
Expt	Cell Line	LPS	none	A10G10	mIgG ₁	none	B5	6F11
1	THP-1	-	3.1	2.7	nd	2.7	34.7	3.4
		+	6.9	16.5	nd	3.7	43.8	3.6
	U937	-	1.6	1.9	nd	1.6	35.7	1.8
		+	3.9	12.7	nd	1.9	43.5	2.6
2	THP-1	-	2.4	3.1	1.6	1.7	17.7	3.4
		+	3.1	8.3	2.2	3.0	29.6	3.2
	U937	-	2.8	2.9	2.8	2.2	20.2	2.7
		+	3.6	11.8	2.4	2.4	28.4	3.2
3	THP-1	-	4.7	6.0*	6.7	4.6	56.3	nd
		+	8.1	4.9*	5.4	5.0	65.9	nd
	U937	-	1.5	9.9*	2.3	1.2	61.4	nd
		+	1.0	13.1*	3.7	0.7	49.3	nd

In all three experiments, LPS increased the amount of B5 binding to THP-1 cells. This was true also for U937 cells in two of the three experiments. In contrast to noninduced cells, LPS stimulation led to the ability to be

1
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3 stained by the A10G10 mAb for both the THP-1 and the U937
4 lines. Nevertheless, the percentages of cells in either
5 line expressing TNF α epitopes recognized by A10G10 were
6 small, in comparison to those percentages seen with the B5
7 mAb. These data suggest that csTNF α can be increased by
8 incubation with LPS and that this increase correlates with
9 the acquisition of TNF α epitopes recognized by neutralizing
10 antibodies.

11
12 Influence of Factors other than LPS on csTNF α Expression.

13 During the course of our experiments, some of our cell
14 lines lost some spontaneous csTNF α expression. To examine
15 the influence of fetal bovine serum (FBS) on csTNF α
16 expression, THP-1 cells were cultivated four days in the
17 different lots of fetal bovine sera and analyzed for cell
18 surface TNF α expression. Table 5 shows typical results.
19 Shown are the percentages of cells staining positively with
20 the indicated primary and fluorescent secondary staining
21 antibodies. The endotoxin concentrations, in Limulus
22 ameocyte lysate units, for FBS lots 1079, 1087, 2081 and
23 1026 are 0.125, 0.250, 0.060 and 0.750, respectively.
24 Analyses were performed with a FACSCAN instrument.
25
26
27
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Table 5

Influence of Fetal Bovine Serum on Cell Surface
Expression of TNF α by THP-1 Cells

		FBS Lot #			
1 st Ab	2 nd Ab	1079	1087	2081	1026
% cells staining positively					
none	none	0.2	0.3	0.1	0.2
none	FL-anti-IgM	2.2	3.5	1.6	2.6
B5	FL-anti-IgM	29.5	15.1	6.8	14.1
6F11	FL-anti-IgM	6.7	7.1	4.2	5.2

The FBS lot had a large influence on csTNF α expression by THP-1 cells. The difference in expression varied by about a factor of four depending on the particular FBS batch used. Comparison of the endotoxin levels in these different lots revealed no direct correlation with csTNF α levels. These data suggest that factors other than LPS can influence expression of csTNF α .

Specificity of B5 mAb Binding to csTNF α

Table 6 presents data which confirm the specificity of B5 mAb binding to the THP-1 cells. B5 mAb at 10 ug/ml was incubated with the indicated concentrations of inhibitors prior to exposure to LPS stimulated THP-1 cells. Its binding was detected with fluorescein conjugated F(ab)'₂ anti-human IgM antibody. LT is recombinant human lymphotoxin, ECD55 is the recombinant extracellular TNF α binding domain of the p55 TNF receptor and A10G10 is the

neutralizing mouse IgG₁ anti-TNF α mAb. Analyses were performed with a FACSCAN instrument.

Table 6
Specificity of B5 anti-TNF α mAb
binding to THP-1 Cell Surface

inhibitor	% Cells Staining Positively				
	0.0	0.03	ug/ml Inhibitor		
			0.30	3.0	30.0
TNF α	44.1	43.2	35.9	22.2	15.8
LT	44.1	39.8	40.0	40.0	29.7
A10G10	44.1	39.6	40.9	44.4	41.9

Preincubation of the B5 IgM mAb with TNF α inhibited its subsequent cell surface binding, in a dose dependent manner, whereas preincubation with lymphotoxin did not, except for a small effect at the highest concentration. The lack of complete inhibition with the high doses of TNF α is consistent with the previously documented low affinity of this mAb for soluble TNF α . Interestingly, preincubation of B5 mAb with A10G10 and subsequent addition of both did not decrease B5 binding. These data suggest that neutralizing A10G10 does not compete for the same epitope on TNF α to which B5 mAb binds.

B5 Binds to csTNF α on Fresh Human Spleen Cells

The previous sections establish B5 binding to csTNF α on several different cell lines. To determine whether or not B5 binds to untransformed cells, experiments were performed with human splenocytes.

To analyze B cell expression of csTNF α by B5, we used unconjugated B5 IgM since direct fluoresceination or biotinylation of this antibody was very inefficient or interfered with its TNF α binding ability. Fluorescent F(ab)'₂ fragments of anti-human IgM antibody were used to detect B5 binding. Since many normal B cells already express sIgM as an antigen receptor, it was not always possible to detect csTNF α as an increase in the percentage of sIgM+ cells. We could, however, detect csTNF α by measuring the increase in staining intensity with the fluorescent anti-IgM when cells are incubated with the B5 mAb compared to incubation with either control 6F11 IgM mAb or no antibody at all.

Figure 12 demonstrates this shift in fluorescence intensity seen when the B5 mAb binds to B cells. Figure 12A shows the fluorescence histogram of cells stained with anti-IgM antibody alone. Figure 12B shows a histogram of these same cells when first reacted with B5 mAb anti-TNF α and subsequently restained with florescent anti-IgM antibody. The most useful statistic to measure this shift is the median channel of fluorescence intensity, or simply median channel. The median channel numbers are presented in the following tables when B cells are examined.

Table 7 presents the data from two experiments using splenic biopsy material. The expression of csTNF α on monocytes, T cells and B cells was examined by two color immunofluorescence analysis using phycoerythrin conjugated anti-LeuM3, anti-CD3 and anti-CD19, respectively, in conjunction with fluorescein conjugated anti-human IgM. Human splenocytes received one day after biopsy were analyzed for expression of cell surface staining with the

indicated monoclonal antibodies. Small lymphocytes were gated by forward and side scatter properties and then analyzed. T cells, B cells and monocytes were stained with phycoerythrin conjugated anti-CD3, anti-CD19 and anti-LeuM3 antibodies, respectively. Two color analyses were then performed on these populations using fluorescein labeled F(ab)'₂ anti-human IgM and the indicated IgM mAbs. Underlined values represent those which show significant increases in the percentage of positively stained cells or show greater than twice the fluorescence intensity of the appropriate control population. Analyses were performed with a FACSCAN instrument.

Table 7
Analysis of Cell Surface TNF α Expression on
Fresh Human Spenocytes

Cells Analyzed	% Cells Staining Positively (median fluorescence intensity channel)				
	1 st Ab: none 2 nd Ab: anti-IgM	B5 anti-IgM	7T1 anti-IgM	H5 anti-IgM	6F11 anti-IgM
<u>Spleen #1:</u>					
lymphocytes	37.9(86)	<u>60.0</u> (246)	<u>44.6</u> (94)	<u>48.0</u> (95)	37.5(88)
CD3+	4.8(21)	<u>28.9</u> (19)	<u>10.5</u> (29)	<u>10.6</u> (22)	3.9(24)
LeuM3+	7.4(125)	<u>28.9</u> (76)	<u>77.1</u> (106)	<u>67.5</u> (124)	8.6(84)
<u>Spleen #2:</u>					
CD3+	8.8(32)	<u>88.3</u> (54)	<u>25.5</u> (46)	13.7(47)	7.1(28)
CD19+	57.5(125)	<u>97.5</u> (910)	<u>71.2</u> (145)	<u>72.2</u> (138)	55.7(124)
Leu-M3+	7.7(196)	<u>49.8</u> (163)	<u>66.8</u> (2272)	<u>58.9</u> (1604)	9.1(173)

In both experiments, monocytes constituted less than 5% of the total splenocyte populations. Of these, a significant fraction in both experiments were stained with the anti-TNF α B5 mAb. On the other hand, these cells were

1
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3 not stained with the control 6F11 human IgM mAb. These
4 results suggest that some splenic monocytes express csTNF α .

5 CD3+ T cells showed variable expression of csTNF α .
6 While the percentages of csTNF α positive T cells varied in
7 these experiments, the staining with the B5 mAb was much
8 weaker than that seen for B cells and monocytes. The median
9 fluorescence intensity for T cell csTNF α was not even twice
10 that seen for the background controls. These results
11 suggest that a variable proportion of splenic T cells
express small amounts of csTNF α .

12 Analysis of B cell csTNF α expression revealed quite
13 strong csTNF α expression. As seen in spleen Z, the
14 percentage of IgM+ B cells increased after incubation with
15 B5 mAb. Furthermore the staining intensity of the entire B
16 cell population approximately tripled. No increase in
17 staining was seen with the 6F11 control antibody, indicating
the specificity of the B5 staining on B cells.

18 The polyreactive mAb 7T1 and H5 were included in these
19 analyses. In addition to binding to TNF α , these antibodies
20 react with several other antigens. Hence the specificity of
21 their cell surface binding is unknown. We include them for
22 comparison not only since they do bind to TNF, but also
23 since little data on binding of polyreactive mAbs to unfixed
24 cells is available. These antibodies do appear to react
25 with T cells and B cells but they react with monocyte
26 surfaces far better. In addition to significant increases
27 in the percentages of B and T cells staining with these
28 antibodies, the majority of monocytes in both experiments
were stained.

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3 These data suggest that the B5 anti-TNF α mAb can react
4 with splenic lymphocytes of the B and T lineages as well as
5 being able to recognize and bind to splenic monocytes.

6 B5 Binding to csTNF α on Cultured Human Spleen Cells

7
8 Spleen cells from one individual examined in Table 7
9 were cultivated in vitro for 3 days with various stimuli and
10 were then analyzed for B5 mAb binding. Results are shown in
11 Table 8. Cultivation of these cells resulted in loss of
12 monocytes so data for Leu-M3+ cells are not presented. The
13 cells were stained for CD3 or CD19 with phycoerythrin
14 conjugated antibodies to allow two color analyses with
15 fluorescein conjugated F(ab)'₂ anti-human IgM and the
16 indicated human IgM mAbs. All cells were analyzed when no
17 activator was included in culture but only large activated
18 cells were analyzed from cultures which included activators.
19 Underlined values represent those which show significant
20 increases in the percentage of positively stained cells or
21 show greater than twice the fluorescence intensity of the
22 appropriate control population. Analyses were performed
23 with a FACSCAN instrument.
24
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Table 8
Analysis of Cell Surface TNF α Expression by
Cultured Human Splenic Lymphocytes

% Cells Staining Positively
(median fluorescence intensity channel)

Activator	Cell	1st Ab	2nd Ab	-	+	B5	7T1	H5	6F11
						+	+	+	+ μ
none	CD3+	0.1(154)	9.3(48)			42.2(17)	11.8(34)	16.6(25)	10.0(57)
	CD19+	1.0(22)	85.1(54)			99.4(294)	91.8(56)	96.1(96)	86.8(52)
anti- δ -Dex plus									
IL-2	CD19+	4.0(76)	96.0(58)			100.0(272)	99.5(82)	99.9(224)	97.4(57)
SEB	CD3+	9.1(110)	24.4(102)			66.4(84)	44.8(82)	53.8(87)	26.1(106)
SAC	CD19+	3.4(42)	58.9(60)			100.0(452)	84.1(93)	94.3(183)	65.6(62)

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3 The cells cultured in medium were 55% CD19+ (B cells)
4 and 22% CD3+ (T Cells). Of the CD19+ cells, 85% were sIgM+
5 with a median channel intensity of 54. Staining with the B5
6 mAb increased this intensity to median channel 294 - nearly
7 six fold higher. This increase was not seen with the
8 polyreactive or control IgM mAbs. Increases in percentages
9 of CD3+ T cells which bound B5 mAb were also seen, although
10 the intensity of staining was low. Despite the fact that
11 anti-IgM alone revealed some T cell staining, addition of
12 6F11 to these T cells did not result in increased anti-IgM
13 staining, showing the specificity of B5 staining and
14 suggesting the B5 mAb is not binding to the IgM receptors
expressed on activated T cells. These receptors are
presumably already occupied and account for the background
staining observed with the anti-IgM secondary antibody.

15 Stimulation with the superantigen Staphylococcal
16 Enterotoxin B (SEB), which activates both T and B cells,
17 resulted in about 24% of the T cells binding the secondary
18 anti-human IgM antibody. However, about 66% of the SEB
19 activated T cells bound B5 anti-TNF α mAb. No increase in
20 sIgM+ T cells was seen with the 6F11 control mAb. These
21 data indicate induction of csTNF α expression when T cells
are activated.

22 B cells activated by either anti-IgD-dextran or
23 Staphylococcus aureus Cowan Strain I (SAC), both potent B
24 cell mitogens, demonstrated binding by B5 anti-TNF α mAb.
25 The higher B5 staining fluorescence intensity seen after SAC
26 induction suggests a higher B cell surface level of TNF α
27 expression than seen on anti-IgD activated B cells, or B
28 cells cultured in medium alone. These data suggest that

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3 both activated human B cells and T cells express csTNF α
4 epitopes recognized by the B5 mAb.

5 Binding of B5 mAb to human and chimpanzee peripheral blood
6 lymphocytes.

7
8 To extend the finding of human splenic lymphocyte
9 expression of csTNF α , peripheral blood lymphocytes of human
10 and chimpanzee origin were examined. Table 9 shows the
11 results obtained with blood from two chimpanzees and one
12 human. The chimpanzee blood was received one day after it
13 was drawn whereas the human blood was fresh. The delay in
14 receipt of the blood appeared to result in loss of monocytes
15 from the chimpanzee blood. Peripheral blood mononuclear
16 cells were prepared by separation on Ficoll and stained with
17 PE derivatized anti-CD3, CD19 or LeuM3. For the chimpanzees
18 171 and 203, less than 2% and 0.6% of cells were LeuM3+,
19 respectively. Some 20.2% of the human cells were LeuM3+. T
20 cells comprised 62% and 54% of the chimpanzee lymphocytes
21 and 68% of the human lymphocytes. B cell percentages were
22 2.8 and 5.4 for the chimpanzees and 16.4% for the human.
23 Cells were incubated with the indicated IgM primary
24 antibodies and subsequently stained with the fluorescein
25 conjugated F(ab)'₂ anti-human IgM reagent. Analyses were
26 performed with a FACSCAN instrument. Underlined values
27 represent those which show significant increases in the
28 percentage of positively stained cells or show greater than
twice the fluorescence intensity of the appropriate control
population.

Table 9

Analysis of Chimpanzee and Human Peripheral Blood
T Cell and B Cell Expression of Cell Surface TNF α

% positive cells (median channel intensity)

primary Ab	-	-	6F11	B5	7T1	H5
anti-IgM	-	+	+	+	+	+
<u>Chimp 171</u>						
CD 3+	0.1(25)	14.4 (40)	14.8 (41)	<u>31.9</u> (23)	18.2 (39)	21.5 (28)
CD19+	0.4(10)	98.6(196)	98.4(196)	99.6(<u>704</u>)	98.9(230)	99.6(312)
<u>Chimp 203</u>						
CD3+	0.0(13)	30.9 (26)	32.1 (27)	<u>53.6</u> (27)	31.2 (25)	42.0 (24)
CD19+	0.6(13)	92.3 (70)	90.1 (79)	99.4(<u>491</u>)	95.1(101)	98.0(196)
<u>Human</u>						
CD3+	0.6(17)	1.7 (24)	3.3 (22)	<u>17.1</u> (15)	2.8 (19)	4.5 (16)
CD19+	1.3(37)	83.5 (75)	84.6 (70)	99.4(<u>316</u>)	91.5 (78)	96.1 (96)
LeuM3+	1.2(26)	5.6 (9)	4.2 (84)	4.8(106)	<u>35.3</u> (74)	<u>30.4</u> (82)

In contrast to the previous results with human spleen, the fresh peripheral human monocytes did not express csTNF α as seen by the B5 mAb. A significant fraction of these cells did, however, bind the polyreactive mAbs 7T1 and H5.

The fresh human T cells did not express surface IgM whereas the chimpanzee T cells drawn one day previously did. T cells from both species, however, expressed modest amounts of csTNF α detected by the B5 mAb. This anti-TNF α staining was very weak, however, and suggests only low levels of csTNF α were present. T cells from neither species were recognized by polyreactive 7T1 or H5.

In contrast to the T cells, peripheral blood B cells from both chimpanzees and the human displayed high levels of csTNF α seen by B5 mAb. This expression was much more

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3 intense than that seen with the T cells. These results
4 suggest that normal human peripheral blood monocytes do not
5 express csTNF α whereas some T lymphocytes and most B
6 lymphocytes from both species do express this cell surface
cytokine.

7
8 B5 anti-TNF α mAb inhibits LPS induced secretion of TNF α
by THP-1 cells.

9
10 To examine whether or not the binding of B5 mAb to
11 csTNF α had any functional significance, we stimulated the
12 THP-1 human monocyte cell line with LPS in the presence of
13 B5 or other human IgM mAbs. We assayed secretion of
14 biologically active TNF α by measuring cytotoxic activity of
15 the supernatants on the TNF α sensitive WEHI 164 cell line.
16 The results of two of four such experiments are given in
17 Table 10. THP-1 cells were stimulated for 4 hours with 100
18 ng/ml E. coli LPS in the presence of 40 ug/ml of the
19 indicated TNF non-neutralizing human IgM mAbs. Supernatants
20 from these incubations were then tested for cytotoxicity
21 against the TNF α sensitive WEHI 164 cell line. All
22 supernatant cytotoxicity was concentration dependent and was
23 neutralized by A10G10 anti-TNF α mAb, indicating cytotoxicity
24 was due to TNF α . Concentrations of secreted TNF α were
25 determined by comparison to a standard curve.
26
27
28

Table 10

Inhibition of LPS Induced TNF α Secretion by B5 mAb

Expt	mAb	ug/ml	pg/ml TNF α	% inhibition
1	none	0	1003	0
	6F11	40	990	1
	7T1	40	976	3
	B5	40	102	90
	"	20	409	59
	"	10	812	19
	"	5	962	4
2	none	0	2057	0
	6F11	40	1992	3
	B5	40	143	93
	"	20	783	62
	"	10	1271	38
	"	5	2276	-10

Stimulated THP-1 cells did secrete active TNF α and all of this cytotoxic activity was inhibited by including A10G10 in the cytotoxicity assay (data not shown). Previous experiments including B5 mAb in the cytotoxicity assay have shown that B5 does not neutralize TNF α (Fig. 9). Table 10 shows that coculture of the THP-1 cells with B5 mAb inhibits LPS induced TNF α secretion. These data suggest that B5 mAb interaction with csTNF α can inhibit LPS induced TNF secretion.

DISCUSSION

To our knowledge, this is the first report of a monoclonal human autoantibody specific for human and mouse $\text{TNF}\alpha$. It is unclear whether or not the CMV seropositive donor origin of B5 mAb is significant. The antibody is clearly different from the mouse mAbs we have generated to $\text{TNF}\alpha$, all of which are neutralizing, as shown previously by Galloway et al (cited above).

Three lines of evidence suggest that B5 mAb recognizes an epitope different from those recognized by the mouse mAbs described. First, there is no competition between the human and mouse mAbs for binding to plates coated with TNF. Second, TNF bound by the human mAb can be recognized by the mouse mAbs, and vice versa. Finally, B5 mAb does not neutralize rh $\text{TNF}\alpha$ whereas the mouse mAbs do. One might argue that $\text{TNF}\alpha$ is a trimer and, as such, $\text{TNF}\alpha$ bound to neutralizing mouse mAbs attached to plates can still present an identical epitope to be recognized by mAb B5. The lack of competition between the mouse mAbs and mAb B5 for plate bound $\text{TNF}\alpha$ is a strong argument against this possibility. The competition data in combination with the lack of neutralizing activity of B5 mAb support the interpretation of distinct epitope recognition by the mouse and human mAbs. The biological effects of $\text{TNF}\alpha$, especially its ability to promote Ig secretion, may preclude the generation of a high affinity neutralizing human anti- $\text{TNF}\alpha$ autoantibody by the techniques used. This ability may also explain the different epitope specificities of B5 mAb and the three neutralizing mouse mAbs.

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2
3 The base of the bell shaped trimeric TNF α molecule,
4 which contains the amino terminus apposed to the carboxy
5 terminus, is the region of the molecule which binds to TNF
6 receptors (M.J. Eck et al "The Structure of Tumor Necrosis
7 Factor- α at 2.6 Å Resolution, Implications for Receptor
8 Binding." J. Biol. Chem. 264:17595-605, 1989; and A. Corti
9 et al "Antigenic Regions of Tumor Necrosis Factor Alpha and
10 Their Topographic Relationships with Structural/Functional
11 Domains." Molec. Immunol. 29:471-9, 1992). Since the mouse
12 mAbs used in this report neutralize TNF α , and have been
13 found to block binding of TNF α to its receptors, it is
14 likely that an epitope in the base of the trimer is
15 recognized by these antibodies. From the data presented in
16 this report, one might speculate that the B5 mAb sees a
17 region of the TNF α molecule closer to the "top" of the
18 trimer.

19
20 The weak binding of B5 mAb to soluble TNF α is
21 consistent with a low binding constant of the mAb for the
22 ligand. Nevertheless, the valency of this IgM mAb can
23 outweigh this shortcoming so that B5 can bind to solid phase
24 TNF α as well as, or better than, the high affinity
25 neutralizing mouse anti-TNF α mAbs tested. Apparently,
26 multipoint binding allows the mAb B5 to adhere strongly to
27 TNF α when a sufficient antigen density is available.

28
29 Although B5 appears to bind with low affinity, we show
30 that it binds specifically to TNF α and fails to bind to any
31 of the other antigens tested. This contrasts with the
32 observed binding of two other control polyreactive mAbs.
33 Hence, B5 appears to be monospecific and is not
34 polyreactive. B5 seems to bind specifically to an epitope,
35 most likely a linear epitope, shared by mouse and human

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3 TNF α . These properties classify B5 as an autoantibody and
4 distinguish it from other mAbs so far described.

5 The human B5 autoantibody binds to surface TNF α on a
6 broad range of human cell lines and lymphoid cells. It is
7 not surprising that it recognizes chimpanzee TNF α as there
8 is no difference in the amino acid sequences of TNF α from
9 chimpanzee and human. We have also shown that B5 recognizes
10 mouse TNF α which is about 80% identical to human TNF α (D.
11 Pennica et al "Cloning and expression in Escherichia coli of
12 the cDNA for Murine Tumor Necrosis Factor", Proc. Natl.
Acad. Sci. USA 82:6060-4, 1985). Hence it is not surprising
that B5 recognizes mouse csTNF α .

13 Others have certainly described TNF production by human
14 B cells (M. Jäätelä, "Biology of Disease. Biologic
15 Activities and Mechanisms of Action of Tumor Necrosis
16 Factor- α /Cachectin", Lab. Invest. 64:724-42, 1991; and
Smeland et al "Interleukin 4 Induces Selective Production of
17 Interleukin 6 from Normal Human B Lymphocytes", J. Exp. Med.
18 170:1463-68, 1989), T cells (S.-S.J. Sung et al Production
19 of Tumor Necrosis Factor/Cachectin by Human T Cell Lines and
Peripheral Blood T Lymphocytes Stimulated by Phorbol
20 Myristate Acetate and Anti-CD3 Antibody", J. Exp. Med.
21 167:937-, 1988), monocytes (Beutler et al "The Biology of
22 Cachectin TNF- α : Primary Mediator of the Host Response",
Ann. Rev. Immunol. 7:625-55, 1989), B cell lines (S.-S.J.
23 Sung et al "Production of Tumor Necrosis Factor/Cachectin by
24 Human T Cell Lines and Peripheral Blood T Lymphocytes
25 Stimulated by Phorbol Myristate Acetate and Anti-CD3
26 Antibody", J. Exp. Med. 167:937-, 1988; and G.J. Jochems et
27 al "Heterogeneity in Both Cytokine Production and
28 Responsiveness of a Panel of Monoclonal Human Epstein-Barr

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3 Virus-Transformed B-Cell Lines", Hum. Antibod. Hybridomas
4 2:57-64, 1991), astrocytes (A.P. Lieberman et al Production
5 of Tumor necrosis Factor and other Cytokines by Astrocytes
6 Stimulated with Lipopolysaccharide or a Neurotropic Virus",
7 Proc. Natl. Acad. Sci. USA, 86:6348-52, 1989; and I.Y. Chung
8 et al "Tumor Necrosis Factor Alpha Production by Astrocytes:
9 Induction by Lipopolysaccharide, IFN-gamma, and IL-1 beta",
10 J. Immunol. 144:2999-3007, 1990; and K. Selmaj et al
11 "Identification of Lymphotoxin and Tumor necrosis Factor in
12 Multiple Sclerosis Lesions", J. Clin. Invest. 87:949-54,
13 1991) as well as some TNF resistant cell lines (B.Y. Rubin
14 et al "Nonhematopoietic Cells Selected for Resistance to
15 Tumor Necrosis Factor Produce Tumor Necrosis Factor", J.
16 Exp. Med. 164:1350-5, 1986). We extend these findings to
17 include at least one metastatic breast carcinoma, DU4475, a
18 melanoma, A375, and the U373 astrocytoma/glioblastoma. We
19 also demonstrate csTNF α expression on human splenic lymphoid
20 cells. This is somewhat surprising since previous
21 demonstration of csTNF α by others tended to employ activated
22 cells.

23
24 Although we examined small lymphocytes, as determined
25 by light scatter properties, it is possible many of these
26 cells were partially activated or at a stage of
27 differentiation where they could express this cell surface
28 molecule. The smaller percentages of T lymphocytes and
monocytes from human peripheral blood expressing csTNF α is
consistent with the resting phenotype of these cells. In
any case, the breadth of csTNF α expression suggests it has
an important role in the surface of many cells.

Others have shown that TNF α can exist as both an
integral transmembrane protein and as a mature protein bound

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3 to its receptor on cell surfaces (B. Luetting et al "Evidence
4 for the Existence of Two Forms of Membrane Tumor Necrosis
5 Factor: an Integral Protein and a Molecule Attached to its
6 Receptor", J. Immunol. 143:4034-38, 1989). Several
7 observations suggest that the B5 mAb recognizes the integral
8 transmembrane protein. B5 binding was increased when cells
9 were activated with LPS or PMA. Both agents, but especially
10 PMA, down regulate TNF receptor expression on a variety of
11 cell types (A.H. Ding et al "Macrophages Rapidly Internalize
12 their Tumor Necrosis Factor Receptors in Response to
13 Bacterial Lipopolysaccharide", J. Biol. Chem. 264:3924-9,
14 1989; and B.A. Aggarwal et al "Effect of Phorbol Esters on
15 Down-Regulation and Redistribution of Cell Surface Receptors
16 for Tumor Necrosis Factor α ", J. Biol. Chem. 262:16450-5,
17 1987).

18 B5 binds to unstimulated cell lines whereas cell lines
19 normally need to be induced to secrete TNF. Hence,
20 unstimulated cell lines would be expected to display little
21 receptor bound TNF. We showed that B5 binding to cell
22 surfaces was inhibited by preincubation with TNF α , but not
23 A10G10 anti-TNF α mAb. This demonstrates the specificity of
24 the B5 antibody.

25 TNF β binds to the same receptors as TNF α and so might
26 compete off some receptor bound TNF α on cell surfaces. The
27 data in Table 6 with high doses of TNF β suggest that this
28 did occur, and was detected by a decrease in B5 staining.
For these reasons, it is likely that B5 recognizes the 26 kd
transmembrane form of TNF α and possibly receptor bound TNF.

One puzzling result of these studies is that B5 mAb
binds to csTNF α in many situations in which A10G10 binding

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3 is either absent or less than that seen with B5. It is
4 clear that these two antibodies see non-overlapping
5 epitopes. Since A10G10 neutralizes TNF α cytotoxicity and
6 prevents TNF α binding to its receptor, this mouse mAb
probably binds to TNF α near the receptor binding domain.

7
8 Others have shown that mAbs which bind the amino
9 terminal 15 or so amino acids block TNF α cytotoxicity (S.H.
10 Socher et al "Antibodies Against Amino Acids 1-15 of Tumor
11 Necrosis Factor Block Its Binding to Cell-Surface Receptor"
12 Proc. Natl. Acad. Sci. USA 84:8829-33, 1987). Hence, it is
13 possible that A10G10 binds to some of the N-terminal amino
14 acids which are most membrane proximal on the transmembrane
15 form of TNF α . This region may not be accessible to A10G10
16 for binding, although the TNF molecule itself is present and
17 can be recognized by B5 mAb.

18
19 Western blotting experiments suggest that A10G10 does
20 not recognize TNF α monomers and probably recognizes a
21 conformational epitope (data not shown). If transmembrane
22 TNF α is primarily monomeric, epitopes recognized by A10G10
23 may not be present. Additional experiments may help to
24 decide between these and other possibilities.

25
26 Interestingly, we did observe A10G10 cell surface
27 binding when cells were activated with LPS. This induction
28 causes secretion of the biologically active TNF α trimer
which can then bind to remaining TNF receptors. Since
trimeric TNF α is multivalent, it may bind to some receptors
in a way which allows one or even two remaining receptor
binding domains to remain free. It may be this form of
cTNF α which is recognized by A10G10. Indeed, others have
shown that incubating unactivated paraformaldehyde-fixed

1 human monocytes with TNF α results in TNF α binding its
2 receptors and renders these monocytes cytotoxic.
3 Furthermore, this cytotoxicity is abolished by neutralizing
4 anti-TNF antibodies (A Nii et al "The Incubation of Human
5 Blood Monocytes with Tumor Necrosis Factor-Alpha Leads to
6 Lysis of Tumor Necrosis Factor-Sensitive but not Resistant
7 Tumor Cells", Lymphokine Res. 9:113-24, 1990).
8

9 One model which explains much of the data is that
10 transmembrane TNF α monomers are recognized by B5 mAb. We
11 have shown soluble monomer recognition by B5. Cell surface
12 TNF α monomers might exhibit an overall conformation
13 different from that of trimeric TNF. They may still expose
14 TNF receptor binding domains and so be capable of mediating
15 cytotoxicity through cell contact. Cells expressing many
16 monomers could thus cause TNF receptor cross-linking on
17 target cells. An activation signal could cause
18 polymerization of TNF monomers in the cell membrane, leading
19 to a conformational change which, in turn, might expose a
20 proteolytic cleavage site leading to release of mature,
21 biologically active trimeric TNF α . Release could be
22 followed by interaction with TNF receptors and allow A10G10
23 binding, as suggested above. B5 apparently binds to
24 membrane distal TNF domains and, by so doing, may interfere
25 with either csTNF α polymerization, a subsequent
26 conformational change, or both. B5 probably does not bind
27 to the proteolytic cleavage site since it does bind to the
28 mature trimeric molecule. This model would explain the cell
surface staining results and also explain the observed
inhibition of TNF secretion after LPS activation of THP-1
cells. It should be noted that this model allows for a role
of the cytoplasmic domain in csTNF α polymerization. This is

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3 only a working model and, as such, is admittedly
4 hypothetical.

5 The invention may be embodied in other specific forms
6 without departing from the spirit or essential
7 characteristics thereof. The present embodiments are
8 therefore to be considered in all respects as illustrative
9 and not restrictive, the scope of the invention being
10 indicated by the appended claims rather than by the
11 foregoing description. All changes coming within the
12 meaning and range of equivalency of the claims are therefore
13 intended to be embraced therein.

14 Given the above examples, it is thought that variations
15 will occur to those skilled in the art. Accordingly, it is
16 intended that the above examples should be construed as
17 illustrative and that the scope of the invention should be
18 limited only by the following claims.
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